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variant expression appears to be developmentally regulated, with the CI cassette-containing splice variants exhibiting increased expression levels around, and immediately after, the time of birth, a time of intense synaptogenesis in the mammalian CNS (17). The role of NR2 subunits in the subcellular localization of NMDA receptors is unknown. The NR2 subunits contain large COOH-terminal domains that are likely to contain additional targeting sequences. Regulation of the cellular distribution, membrane density, and cytoskeletal interactions of NMDA receptors at the synapses may prove to be important processes in both synaptogenesis and in long-term alterations of synaptic efficiency that underlie synaptic plasticity.

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20. The cDNAs for NR1A, NR1C, NR1D, NR1E, mutant NR1A S889A S890A, mutant NR1A S896A S897A, GluR1, GluR6, NGluR1-Cterm, and NGluR1-$(CI)_2$, subcloned into an expression vector containing the cytomegalovirus promoter, were transfected into QT6 cells by calcium phosphate coprecipitation with the use of 2 μg of DNA per well in two chambered glass chamber slides (Nunc) or 20 μg per 10-cm culture dish. Approximately 40 to 48 hours after transfection, the cells were processed for immunofluorescent staining or solubilized for immunoblot analysis.
21. The antibodies used in these experiments include affinity-purified guinea pig antibody to GluR1 (1:300 dilution) (2, 27), rabbit antibody to GluR6 (1:300 dilution) (27), and rabbit antibody to NR1 (1:1000 dilution) (18). Additionally, an affinity-purified rabbit antibody to NR1 splice variants lacking the second COOH-terminal exon cassette (CII) was generated against the synthetic peptide KRRAIEREEGQLQLCSRHRES (where K, Lys; R, Arg; A, Ala; I, Ile; E, Glu; G, Gly; Q, Gln; L, Leu; C, Cys; S, Ser; H, His) corresponding to the COOH-terminus of NR1D and NR1E (1:1000). For immunofluorescent staining, transfected cells were rinsed with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS containing 4% sucrose for 15 min at room temperature (RT), permeabilized with 0.25% Triton X-100 in PBS for 5 min at RT, blocked with 10% goat serum in PBS for 1 hour at 37°C, incubated with primary antibody in PBS containing 3% goat serum for 1 hour at RT, incubated with secondary antibody in PBS containing 3% goat serum for 1 hour at RT, and then mounted with use of Vectastain fluorescent mounting media (Vector). Stained cells were visualized with a Zeiss Axiophot fluorescence microscope equipped with rhodamine and fluorescein optics.
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23. Immunoblots were performed as described (27) (M. D. Ehlers, W. G. Tingley, R. L. Huganir, data not shown).
24. Optical sections (1 to 2 μm thick) in the *x-y* plane were obtained by scanning laser confocal microscopy with an argon laser and the Bio-Rad MRC-600 confocal microscope system. Excitation was at 488 nm, and emissions were taken between 510 and 515 nm. Images were obtained and processed with COMOS version 6.03 (Bio-Rad).
25. M. D. Ehlers, W. G. Tingley, R. L. Huganir, data not shown.
26. Site-directed mutagenesis was used to introduce a silent mutation in the GluR1 cDNA, converting the third nucleotide from the Ala^{891} codon from a C to a T with the use of the antisense primer 5′-CTCCTCCGCTAGCTCCTG-3′. This mutation introduced a unique Nhe I restriction site in the COOH-terminal domain of the GluR1 cDNA, which was used as an insertion site for NR1 DNA fragments amplified by the polymerase chain reaction (PCR). The NGluR1-Cterm chimera was generated by insertion of the NR1 DNA fragment obtained by PCR amplification from NR1 cDNA with the sense primer 5′-GATCATGATGCTAGCGAGATCGCCTACAAGCGAC-3′, and the antisense primer 5′-CATGATCATGCTAGCGCTCTCCCTATGACGGGAACACAG-3′, corresponding to amino acids 834 to 938 of NR1 into the introduced Nhe I site of the GluR1 cDNA. The NGluR1-$(CI)_2$ chimera was generated in a similar manner with the sense primer 5′-GATCATGATGCTAGCAGGAAGAACCTGCAGGATAG-3′ and the antisense primer 5′-CATGATCATGCTAGCGGTGCTCGTGTCTTTGGAGG-3′, corresponding to amino acids 859 to 902 of NR1. Nucleotide sequences of the chimeric receptor constructs were verified by DNA sequencing.
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28. The NR1 cDNAs were provided by S. Nakanishi and the GluR1 and GluR6 cDNAs by S. Heinemann. This research was supported by the Howard Hughes Medical Institute (R.L.H.) and the Medical Scientist Training Program (M.D.E. and W.G.T.). Special thanks go to C. L. Pinch for assisting in the preparation of the manuscript.

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Domain Interaction Between NMDA Receptor Subunits and the Postsynaptic Density Protein PSD-95

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The *N*-methyl-D-aspartate (NMDA) receptor subserves synaptic glutamate-induced transmission and plasticity in central neurons. The yeast two-hybrid system was used to show that the cytoplasmic tails of NMDA receptor subunits interact with a prominent postsynaptic density protein PSD-95. The second PDZ domain in PSD-95 binds to the seven–amino acid, COOH-terminal domain containing the terminal *t*SXV motif (where S is serine, X is any amino acid, and V is valine) common to NR2 subunits and certain NR1 splice forms. Transcripts encoding PSD-95 are expressed in a pattern similar to that of NMDA receptors, and the NR2B subunit co-localizes with PSD-95 in cultured rat hippocampal neurons. The interaction of these proteins may affect the plasticity of excitatory synapses.

NMDA receptors comprise a family of ionotropic glutamate receptors (*1*) with properties that indicate they have a central role in synaptic plasticity and memory formation (*2*). These receptors are formed by assembly of the principal subunit NR1 (*3*) with different modulatory NR2 subunits (NR2A-D) (*4*). A conspicuous structural feature of the NR2 subunits is their extended, intracellular COOH-terminal sequence distal to the last transmembrane region (*5*), which may anchor the receptors or assemble a signal-transducing complex for the voltage-dependent Ca^{2+} entry through the glutamate-activated ion channel (*6*). Thus, we set out to identify intracellular proteins that bind to the NR2 subunits at synapses.

We used the two-hybrid system (*7*) to identify such cellular targets for the COOH-terminal domain of NR2 subunits. A bait consisting of the yeast *GAL4* DNA binding domain (amino acids 1 to 147) fused to the entire COOH-terminal domain (627 amino

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acids) of NR2A (8) was expressed from a shuttle vector introduced into yeast strain YPB2 (9). This strain was transformed with a rat brain complementary DNA (cDNA) expression library (10) constructed in plasmid pGAD (9) to produce proteins tagged on the NH_2-terminus with the *GAL4* activation domain. Library clones activating the expression of both the selection marker *HIS3* and the *LacZ* reporter gene were sequenced. Our screen identified a plasmid (pGAD-PSD) with nearly the entire coding region (10) for the prominent postsynaptic density protein PSD-95 (11, 12).

To determine whether other NR2 subunits also interact with PSD-95, we constructed baits consisting of COOH-terminal NR2 sequences tagged with the *GAL4* DNA binding domain. These were tested for reporter gene activation after cotransfection of yeast with pGAD-PSD. We found that COOH-terminal sequences of NR2B (Fig. 1A) and NR2D (8) also interacted with PSD-95. Dissection of the COOH-terminal NR2B sequences (Fig. 1A) revealed that activation of reporter genes depended on the presence of the seven amino acids at the COOH-terminus. Indeed, a bait constructed from synthetic DNA encoding only the COOH-terminal seven residues (8) showed activity, identifying these residues as those that mediate the interaction of NR2B with PSD-95. These residues are conserved (Fig. 1A) in the otherwise divergent cytoplasmic tails of the NR2 subunits (4). A similar sequence (PSVSTVV) (13) occurs at the COOH-termini of NR1 splice forms NR1-3 and NR1-4 (14), which also interacted with PSD-95. Thus, it appears that the COOH-terminal domains, characterized by a sequence that we termed the tSXV motif (15), confer on NMDA receptors the ability to interact with PSD-95.

PSD-95 (11) is a multidomain protein with three PDZ repeats (16), a Src homology (SH3) domain, and a 190–amino acid sequence having homology to yeast guanylate kinase. To identify which part of PSD-95 interacts with the tSXV domain, we constructed a library of fusion proteins of the *GAL4* activation domain with ~500-bp DNA fragments randomly generated by sonication of pGAD-PSD (17). This tagged fragment library was transfected into yeast together with a vector encoding the tSXV sequence of NR2B appended to the *GAL4* DNA binding domain, and yeast colonies were selected by histidine starvation. DNA from nine selected colonies was amplified by the polymerase chain reaction (PCR) with plasmid-specific primers, and the amplified DNAs were sequenced. All sequences shared the entire PDZ2 coding region (Fig. 1B), which indicates selectivity of the NR2B tSXV motif for PDZ2 as well as a requirement for the complete PDZ domain. This selectivity was maintained when the three PDZ domains were expressed separately (17). Furthermore, the NR2B tSXV motif failed to bind to the single PDZ domain of neuronal nitric oxide synthase (11). Selective interaction with PDZ2 was also observed for the tSXV domains of NR2A, NR2C/D, and NR1-3/4 (8), which indicates that all NMDA receptor subtypes (4) can bind PSD-95 by means of PDZ2 in spite of the sequence variation in their tSXV motifs.

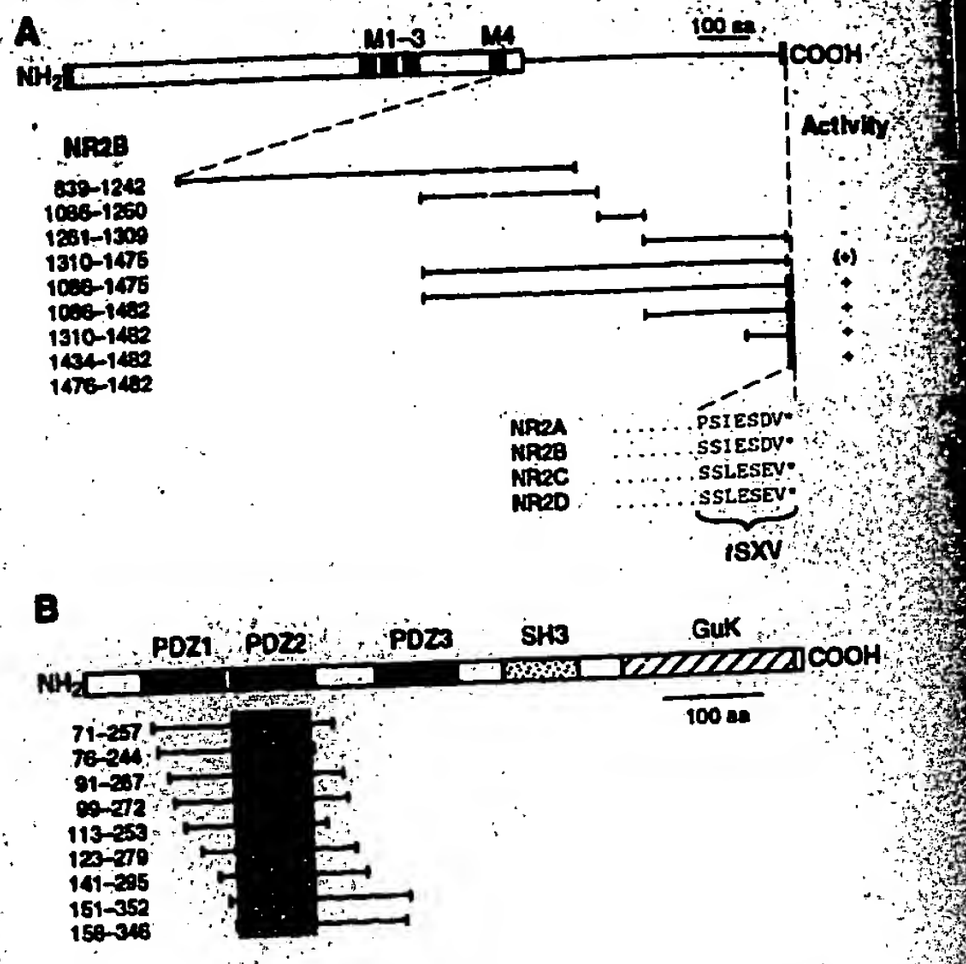

Fig. 1. Interaction of NMDA receptor NR2 subunits (4) and PSD-95 (16). (**A**) The NR2 subunits contain a conserved COOH-terminal sequence that binds to PSD-95. A map of NR2 subunits includes the signal sequence (shaded) and regions of membrane insertion (filled boxes, M1–4). The COOH-terminal regions in NR2A-D differ in sequence and length, as denoted by the single line. Shown below are segments of the COOH-terminal region of NR2B appended to the *GAL4* DNA binding domain (8), specified by amino acid (aa) numbers on the left with the methionine of the immature polypeptide as the first residue. The ability of these fusion proteins to activate the selection marker *HIS3* and the reporter gene *LacZ* upon cotransfection with pGAD-PSD in yeast is listed on the right. Low levels of activity that could not be traced to a particular sequence are indicated by (+). Strong activation required the COOH-terminal seven amino acids containing the tSXV motif (15), which are shown for NR2A-D in the single-letter code (13), with the asterisk denoting the stop codon. (**B**) The tSXV domain of NR2B interacts with the second PDZ domain in PSD-95. The map of PSD-95 specifies the three PDZ domains, an SH3 domain, and the guanylate kinase region (GuK). Randomly generated PSD-95 sequences interacting in the yeast two-hybrid system with the COOH-terminal tSXV sequence of NR2B (17) are aligned to the domain map and identified by amino acid numbers. All selected clones shared the PDZ2 coding sequence (shaded).

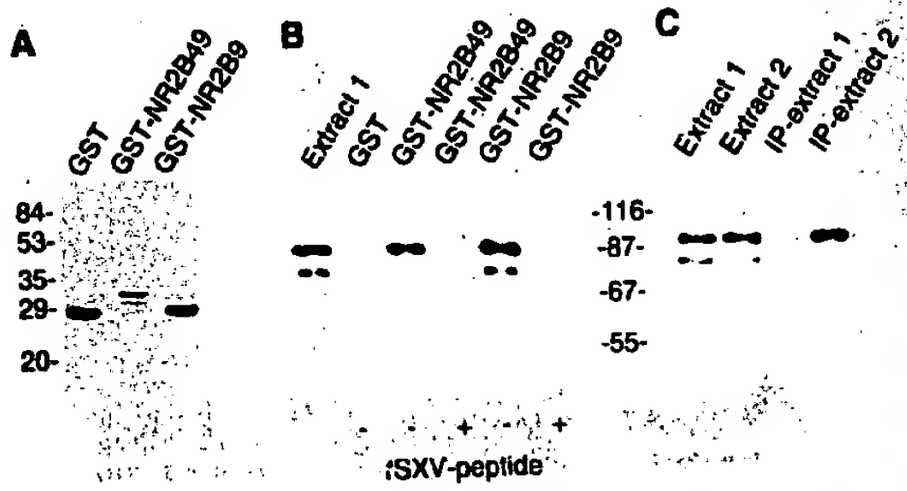

Fig. 2. In vitro and cellular binding of NR2B COOH-terminal sequences to PSD-95. (**A**) GST fusion proteins (18), resolved by SDS–polyacrylamide gel electrophoresis and stained with Coomassie blue. Molecular size markers are indicated on the left in kilodaltons. DNAs encoding the COOH-terminal 49 and 9 amino acids of NR2B were inserted into pGEX-2T (Pharmacia) in-frame with the GST moiety. The fusion proteins expressed in *Escherichia coli* DH5α after induction by isopropyl-β-D-thiogalactopyranoside were purified on glutathione-Sepharose beads (18). (**B**) Immunoblots (20) of PSD-95 recovered by interaction with GST, GST-NR2B49, and GST-NR2B9 bound to glutathione-Sepharose beads from cytoplasmic extracts of HEK 293 cells expressing PSD-95 (19) (extract 1). The first lane contains 1/20 of the amount of extract 1 used in affinity precipitations. Where indicated, a 100-fold excess of a tSXV motif containing peptide was present during incubation. (**C**) Co-immunoprecipitation of the COOH tail of NR2B and PSD-95 from co-transfected HEK 293 cells (21). Shown are immunoblots (20) detecting PSD-95 in NR2B immunoprecipitates (IP) of extract 1 (containing only PSD-95) and extract 2 (containing recombinantly expressed PSD-95 and a 397-residue NR2B COOH-terminal sequence). Equal amounts of extracts were analyzed in parallel to confirm the similar concentrations of PSD-95 (extract 1 and extract 2). Molecular size markers (in kilodaltons) are shown for both (B) and (C).

We sought independent evidence for the interaction of NR2B COOH-terminal sequences with PSD-95. Fusion proteins of glutathione-S-transferase (GST) coupled with the COOH-terminal 49 residues or 9 NR2B residues were bound to glutathione-coupled Sepharose beads (18) (Fig. 2A) and were incubated with HEK 293 cell extracts containing full-length recombinant PSD-95 (19). Protein bound to the GST-NR2B fusion proteins was resolved on SDS-polyacrylamide gels and probed with a mono-

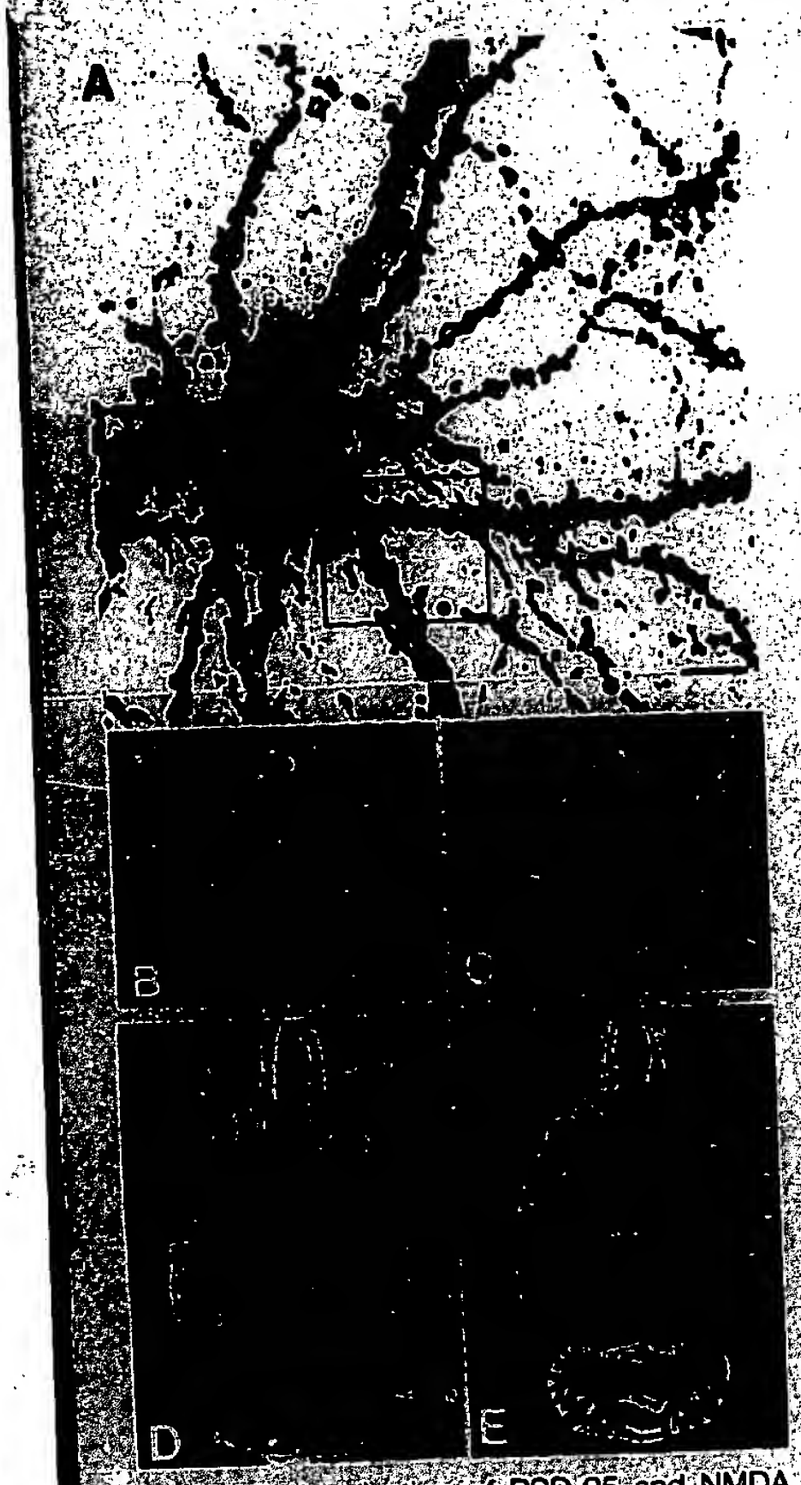

Fig. 3. Co-localization of PSD-95 and NMDA receptor subunits in hippocampal cultures and co-expression of PSD-95 and NMDA receptor subunits in the brain. (**A**) Double image of a hippocampal neuron fluorescently labeled (*23*) with antibodies against both NR2B (green channel) and PSD-95 (red channel). Yellow indicates overlapping fluorescence. Pixels darker than 82% K (where K designates black in the CYMK color system) were changed to white to improve contrast. Bar equals 10 μm. (**B** and **C**) Enlarged single images of the labeling patterns of antibody to PSD-95 (B) and antibody to NR2B (C) from the boxed region in (A). Note the close registration of brightly labeled spots along dendrites. Bar equals 5 μm. (**D** and **E**) In situ hybridization (*24*) in horizontal sections of rat brain (P30) for transcripts encoding PSD-95 (D) and NR1 (E), the principal subunit present in all native NMDA receptor channels (*3*, *4*). OB, olfactory bulb; Cx, cortex; Hi, hippocampus; Cb, cerebellum.

Table 1. Different receptor and channel proteins carry a *t*SXV motif (*13*). Entries were retrieved from the SwissProt R30 database by a search with Inherit Analysis (Perkin-Elmer) and S/TXV* (*15*) as a query.

Protein	Accession no.	*t*SXV sequence
Glutamate receptors		
NMDAR2B (rat)	Q00960	SSIESDV
NMDAR1-3/4 (rat)	P35439	PSVSTVV
GluR (*Lymnaea*)	P26591	SNTHTEV
K^+ channels		
Shaker A/B (*Drosophila*)	P08510/11	VSIETDV
RCK1 (rat)*	P10499	SKLLTDV
Na^+ channels		
α subunit, electric organ (eel)	P02719	VVRESIV
α subunit, cardiac muscle (rat)	P15389	RDRESIV
α subunit, skeletal muscle (rat)	P15390	GVKESLV
G protein–coupled receptors		
β_1 adrenoreceptor (rat)	P18090	FSSESKV
Serotonin receptor 2A (rat)	P14842	NEKVSCV
Serotonin receptor 2C (rat)	P08909	SERISSV
VIP receptor (rat)	P30083	QAEVSLV
CRF receptor (rat)	P35353	IKQSTAV
Mas (rat)	P12526	VSIETVV
Other receptors		
Toll (*Drosophila*)	P08953	NAKQSDV
Fas (human)	P25445	NEIQSLV
NGF receptor, p75 (rat)	P07174	STATSPV
Fasciclin II (*Drosophila*)	P34082	IGKNSAV
NR-CAM (chicken)	P35331	NAMNSFV
V-CAM (rat)	P29534	EAQKSKV

*RCK2 to 5 also carry *t*SXV sequences.

clonal antibody to PSD-95 (*20*). Both GST-NR2B fusion proteins bound PSD-95 to the same extent, whereas no PSD-95 was recovered by GST alone (Fig. 2B). Furthermore, the presence of a synthetic peptide containing the *t*SXV domain of NR2B prevented PSD-95 binding to the GST-NR2B fusion proteins (Fig. 2B), whereas no interference was observed with a control peptide (*19*). We also found that the NR2B–PSD-95 complex could be immunoprecipitated from extracts of HEK 293 cells cotransfected with expression vectors for PSD-95 and the COOH-terminal region of NR2B (*21*), which indicates formation of the binary complex in a cellular environment (Fig. 2C).

Both the NMDA receptor NR2B subunit and PSD-95 are highly enriched in the postsynaptic density fraction from rat brain (*11*, *22*). They were found to co-localize when cultured hippocampal neurons were double-labeled with antibodies (*20*, *23*) for each protein (Fig. 3, A through C). Both proteins were concentrated along dendrites at putative synaptic sites in dense clusters that had virtually identical shape and size. Furthermore, in situ hybridization in rat brain (*24*) demonstrated that PSD-95 transcripts are highly expressed in most neuronal populations (Fig. 3D), as are transcripts for NMDA receptors (*3*, *4*) (Fig. 3E). Thus, these proteins have the potential to interact in most, if not all, central glutamatergic synapses. We postulate that partnership between PSD-95 and NMDA receptors is important in the assembly of multiprotein complexes involved in NMDA receptor–mediated synaptic plasticity (*2*).

Our data identify the *t*SXV and PDZ domains as modular protein binding interfaces. The PDZ domain occurs singly or multiply in a family of intracellular proteins, most of which are localized at specialized sites of cell-to-cell contact, including synaptic sites as well as septate and tight junctions (*16*). It appears that this domain serves in the assembly of submembranous protein complexes with diverse functions. A database search retrieved numerous other receptors and channel proteins with a *t*SXV motif (Table 1). Although interaction of these with proteins carrying PDZ domains remains speculative at this time, members of a voltage-gated K^+ channel family present a likely case, reminiscent of that documented here for NMDA receptors. Rat RCK subunits encoded by distinct genes (*25*) terminate in similar *t*SXV sequences, and splice variants of the homologous *Drosophila Shaker* gene (*26*) with divergent COOH-terminal sequences converge in an identical *t*SXV sequence. We propose that *t*SXV-PDZ domain interactions play a general role in connecting receptors and channels to signal transduction machineries (*27*).

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8. A cloned cDNA encoding the COOH-terminal 627 residues of NR2A (*4*) was inserted into pGBT9 (*9*) to yield pGBT-NR2A, which encodes a fusion protein of the *GAL4* DNA binding domain with the NR2A COOH-terminus. Similar fusion proteins were generated with PCR-generated DNAs encoding segments of the NR2B COOH-terminus (see Fig. 1A), with the NR2D COOH-terminal 180 residues, and with synthetic DNA encoding different tSXV sequences.
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10. A directed cDNA library (pGAD-lib) was prepared in pGADGH (*9*) from rat brain polyadenylated RNA. Briefly, reverse transcription was performed with a random primer having a 5′ Xho I site (5′-GAGAGCTCTCCTCGAGCNNNNNNN-3′), and Eco RI adaptors (Rad1: 5′-AATTCCGAGACGCGTC-3′; Rad2: 5′-GACGCGTCTCGG-3′) were added to the blunt-ended cDNA. The cDNA was digested with Xho I, and molecules >400 bp were inserted into pGADGH. Electroporation into *Escherichia coli* strain DH5α generated 2 × 10^6 clones; 60% of the plasmids (pGAD) contained inserts (average size, 600 bp; range, 400 to 2000 bp). Yeast strain YPB2 (*9*), transformed to harbor pGBT-NR2A, was transformed with pGAD-lib, yielding 6 × 10^6 colonies that were plated on media containing 25 mM 3-aminotriazole, selective for transcriptional activation of the *HIS3* gene [P. L. Bartel, C. T. Chien, R. Sternglanz, S. Fields, *Cellular Interactions In Development: A Practical Approach*, D. A. Hartley, Ed. (Oxford Univ. Press, Oxford, 1993), pp. 153–179]. Colonies grown after 5 days were assayed for β-galactosidase expression. pGAD plasmids from double-positive clones were retransformed into YPB2 together with pGBT, pGBT-NR2A, and pGBT-SNF1 (*7*) to check for specificity. pGAD-PSD contained the coding region for PSD-95 lacking 55 NH_2-terminal and 5 COOH-terminal codons.
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13. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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15. The COOH-terminal consensus sequence in NR2 subunits is (P,S)S(I,L)ES(D,E)V* (*13*), with the asterisk denoting a stop codon. With NR1-3 and NR1-4, the consensus sequence reduces to (P,S)S(L,I,V)X-(S,T)XV*. Reiterative library searches best retrieved other receptors and channels with similar COOH-terminal sequences (Table 1, standard single-letter code), with (S,T)XV* as a query. This terminal consensus was designated as the tSXV motif, and S and T invariance is compatible with phosphorylation.
16. PDZ domains span approximately 90 residues and were first defined as a series of repeats in PSD-95 (GLGF repeats) (*11*, *13*). The PDZ domain is present in several homologous proteins, including discs large, a *Drosophila* septate junction protein [D. F. Woods and P. J. Bryant, *Cell* **66**, 451 (1991)]; Z01, a mammalian tight junction protein [M. Itoh *et al.*, *J. Cell Biol.* **121**, 491 (1993)]; h-dlg [R. A. Lue, S. M. Marfatia, D. Branton, A. H. Chishti, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9818 (1994)]; SAP-97, the rat version of h-dlg [M. Müller *et al.*, *J. Neurosci.* **15**, 2354 (1994)]; as well as in a number of signal transduction molecules of undetermined subcellular location [C. F. Ponting and C. Phillips, *Trends Biochem. Sci.* **20**, 102 (1995)], including nitric oxide synthase (*11*). Also referred to as a DHR domain (discs large homology region), it has now been named a PDZ domain (PSD-95, discs large, Z01).
17. pGAD-PSD with a mutated Eco RI site was sonicated to an average size of 500 bp, and blunt-ended DNA fragments were cloned into the Eco RV site of pBS-SK⁻. The inserts of 10,000 recombinant plasmids retrieved by Eco RI–Sal I digestion were directionally cloned into pGAD. The resulting library was transformed into yeast strain YPB2 carrying pGBT-tSXV of NR2B. Transformants selected for *HIS3* expression were picked after 5 days, and cloned DNA sequences were amplified by PCR with primers *GAL4* AD3′ (5′-AAGAGATCCTAGAACTAGTG-GATC-3′) and T7 (5′-CGTAATACGACTCACTAT-AGGGCG-3′) flanking the multicloning site of pGAD. The sequences of the purified amplified DNAs were aligned to PSD-95. The individual PDZ domains in PSD-95 and neuronal nitric oxide synthase (*11*), PCR-amplified and cloned in pGAD, were tested for reporter gene activation in YPB2 co-transformed with pGBT-cloned tSXV domains from the different NMDA receptor subunits (*8*).
18. D. B. Smith and K. S. Johnson, *Gene* **67**, 31 (1988).
19. The cloned full-length cDNA of PSD-95 in a eukaryotic expression vector [T. J. Schall *et al.*, *Cell* **61**, 361 (1990)] was transfected into HEK 293 cells. After 48 hours, the cells were resuspended in phosphate-buffered saline (PBS) and lysed by Ultraturrax (15 s, 4°C), and membranes and nuclei were pelleted (1 hour, 100,000*g*). Aliquots of the supernatant (extract 1) containing 150 μg of protein were diluted with PBS to a final volume of 300 μl and incubated (1 hour, 37°C) with glutathione-Sepharose beads saturated with GST fusion protein or GST, in the presence or absence of 0.5 mM tSXV peptide (KLSSIESDV) (*13*) or control peptide (CSKDTMEKSESL) (*13*). The beads were washed three times with 1 ml of PBS and 0.1% Triton X-100, and bound protein was resolved by SDS–polyacrylamide gel electrophoresis [U. K. Laemmli, *Nature* **227**, 680 (1970)]. Nitrocellulose blots of gels were probed (12 hours, 4°C) with monoclonal antibody 7E3-1B8 (1:500) (*19*) to PSD-95.
20. Mouse monoclonal antibodies 7E3-1B8 (used for immunoblots) and 6G6-1C9 (used for immunohistochemistry) were selected [G. Köhler and C. Milstein, *Nature* **256**, 495 (1975)] after immunization with recombinant PSD-95 protein (*11*). They recognize the same doublet of proteins on protein immunoblots of brain homogenates as that recognized by an affinity-purified rabbit antiserum to PSD-95. Mouse monoclonal antibody 1B3.3B6 (Boehringer Mannheim) was isolated after immunization against NR2B residues 1325 to 1461 (D. J. Laurie, I. Bartke, K. Naujoks, P. H. Seeburg, unpublished results). Rabbit antiserum to the COOH-terminus of NR2B was prepared by immunization with a fusion protein between GST and NR2B residues 1149 to 1482. This serum (1:2000) recognizes a single, 180-kD band on protein immunoblots of rat brain homogenates.
21. HEK 293 cells were co-transfected with vectors for full-length PSD-95 and a 397-residue NR2B polypeptide of the COOH-terminus. Cytoplasmic extracts from these cells (extract 2) and from cells expressing PSD-95 (extract 1) were diluted in PBS. The NR2B antibody 1B3.3B6 (1:50) (*20*) was added to both extracts (2 hours, 4°C). Incubation was continued for 5 hours in the presence of protein A–Sepharose beads; beads were washed, and bound protein was resolved by SDS gel electrophoresis. Gel blots were analyzed for PSD-95 with a monoclonal antibody (*20*).
22. I. S. Moon, M. L. Apperson, M. B. Kennedy, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3954 (1994).
23. For immunocytochemistry, cultured E18 rat hippocampal neurons [G. J. Brewer, J. E. Torricelli, E. K. Evege, P. J. Price, *J. Neurosci. Res.* **35**, 567 (1993)] were rinsed with PBS, fixed in cold methanol (−20°C, 20 min), and rinsed in PBS and then in preblock (*11*). Double-stained cultures were incubated with antibody to PSD-95 (6G6-1C9, 60 μg/ml) and rabbit antiserum to NR2B (1:500) (*19*), rinsed, and incubated for 1 hour with a mixture of Cy3 goat antibody to mouse immunoglobulin G (IgG) (Cappel, Organon-Teknika) and fluorescein goat antibody to rabbit IgG (Chemicon), each at 1:100 dilution. After the final washes, cultures were scanned in a Zeiss LSM 310 laser-scan confocal microscope through a 63× oil immersion lens at 64 s and at Zoom 2. Cy3 was excited at 543 nm and fluorescein at 488 nm. Images were recorded through standard emission filters at contrast settings for which the crossover between the two channels was negligible, then combined and displayed with Photoshop software. Images from cultures in which the dye labels were reversed showed the same co-localization. Preabsorption of the primary antibodies with their respective antigen proteins (*20*) eliminated the bright staining of discrete spots along the dendrites. Some diffuse labeling of somas and dendrites remained in the cultures labeled with preabsorbed antibody to NR2B, which indicates that labeling of somas and dendritic shafts is partially nonspecific. Images recorded after incubation of cultures with secondary antibodies alone resembled images obtained with preabsorbed primary antiserum.
24. In situ hybridization [W. Wisden, B. J. Morris, S. P. Hunt, *Molecular Neurobiology: A Practical Approach*, J. Chad and H. Wheat, Eds. (IRL Press, Oxford, 1991), pp. 205–225] was performed in horizontal sections of postnatal day 30 (P30) rats with antisense oligonucleotides [5′-CCCCTTTCCAATGTGATCTC-CTCATACTCCA-TCTC-3′ and 5′-GTTTATACTGAG CGATGATCGTGACCGTCTGACCC-3′] for PSD-95 codons 60 to 70 and 383 to 393. These yielded identical patterns and had 12 and 9 mismatches, respectively, to the sequence-related rat SAP-97 cDNA (*16*), which precluded cross-hybridization under the conditions used. The pattern for the principal subunit NR1 (*3*) generated by the NR1-pan oligomer [D. J. Laurie and P. H. Seeburg, *J. Neurosci.* **14**, 3180 (1994)] resembles that of the combined NR2 subunit expression (*4*).
25. W. Stühmer *et al.*, *EMBO J.* **8**, 3235 (1989).
26. T. L. Schwarz, B. L. Tempel, D. M. Papazian, Y. N. Jan, L. Y. Jan, *Nature* **331**, 137 (1988).
27. T. Sato, S. Irie, S. Kitada, J. C. Reed, *Science* **268**, 411 (1995).
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